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Mac-1 Signaling via Src-Family and Syk Kinases Results in Elastase-Dependent Thrombohemorrhagic Vasculopathy

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Summary

CD18 integrins promote neutrophil recruitment, and their engagement activates tyrosine kinases, leading to neutrophil activation. However, the significance of integrin-dependent leukocyte activation in vivo has been difficult to prove. Here, in a model of thrombohemorrhagic vasculitis, the CD18 integrin Mac-1 on neutrophils recognized complement C3 deposited within vessel walls and triggered a signaling pathway involving the Src-family kinase Hck and the Syk tyrosine kinase. This led to neutrophil elastase release, causing hemorrhage, fibrin deposition, and thrombosis. Mice genetically deficient in any of these components (C3, Mac-1, Hck, Syk, or elastase) were resistant to disease despite normal tissue neutrophil accumulation. Disease was restored in Mac-1-deficient mice infused with wild-type, but not kinase- or elastase-deficient, neutrophils. Elastase release in the inflamed tissue was reduced in Mac-1-deficient mice, and a deficiency of Mac-1 or the kinases blocked neutrophil elastase release in vitro. These data suggest that Mac-1 engagement of complement activates tyrosine kinases to promote elastase-dependent blood vessel injury in vivo.

Introduction

Neutrophils are key players of the innate immune response during both host defense and inflammation. Neutrophil functions are largely dependent on the leukocyte-specific CD18 integrins. In vivo, blockade or a deficiency of CD18 integrins limits inflammation, primarily by reducing neutrophil recruitment to the site of injury. In vitro, engagement of CD18 integrins signals activation of effector functions (ROS production and degranulation) (Harris et al., 2000). However, despite the clear evidence that neutrophil integrin signaling in vitro leads to cellular activation, it remains unclear to what extent this contributes to tissue injury or host defense in vivo. Indeed, in immune complex-mediated inflammatory diseases, it is often assumed that signaling through neutrophil Fc receptors is primarily responsible for activation of cellular effector function, while the integrins serve mainly a supporting role to facilitate cellular recruitment. Given the complexity of most disease models, where infiltrating inflammatory cells are receiving signals through numerous ligands, it is often difficult to sort out the relative contribution of integrins alone to neutrophil activation in vivo. Thus, defining a model of an inflammatory disease that is strictly dependent on integrin signaling of neutrophil effector functions remains an elusive goal in the field of inflammation research.

The CD18 integrins LFA-1 (CD11a/CD18, $\alpha_L\beta_2$), Mac-1 (CD11b/CD18, $\alpha_m\beta_2$, CR3), p150,95 (CD11c/CD18, $\alpha_c\beta_2$), and CD11d/CD18 ($\alpha_d\beta_2$) are composed of unique CD11 subunits complexed to a common CD18 subunit. Patients with a deficiency in the CD18 subunit have increased susceptibility to bacterial and fungal infections, which is associated with the inability of their neutrophils and macrophages to migrate and perform cytotoxic functions (Anderson and Springer, 1987). In mice, a deficiency in CD18 or a CD18 receptor intercellular adhesion molecule 1 (ICAM-1), present on the endothelium and leukocytes, attenuates neutrophil accumulation and associated tissue injury in models of inflmmation (Harris et al., 2000; Kakkar and Lefer, 2004). Studies in mice deficient in LFA-1 suggest that it plays a dominant role in neutrophil vascular arrest and transendothelial migration (Andrew et al., 1998; Ding et al., 1999; Henderson et al., 2003). In contrast, Mac-1, which is the predominant CD18 integrin on neutrophils, is postulated to play a more important role in activation of effector functions (Coxon et al., 1996; Ding et al., 1999; Lu et al., 1997; Mayadas and Cullere, 2005; Tang et al., 1997).

Mac-1-mediated adhesion to complement iC3b-coated microbes promotes neutrophil phagocytosis and the release of oxygen radicals and proteinases within phagolysosomes, which is important for host defense. However, when the opsonized target is too large to ingest, the extracellular release of these products may contribute to tissue injury during inflammation (Arnaout, 1990; Ross, 2000). In vitro studies have delineated signaling cascades responsible for CD18 integrin-mediated adhesion, degranulation, and reactive oxygen species (ROS) generation (Lowell, 2004; Lowell and Berton, 1999). Integrin engagement results in activation of Src-family and Syk tyrosine kinases, which colocalize with CD18 integrins to phosphorylate a number of downstream targets including Cbl, Pyk-2, PLCγ1 and 2, ERK, Vav, and Paxillin. Of these substrates, Cbl, SLP-76, and Vav 1,3 have been demonstrated to play a role in neutrophil and macrophage integrin signaling in vitro (Caveggion et al., 2003; Gakidis et al., 2004; Newbrough et al., 2003). In vitro neutrophils deficient in Src-family members Hck and Fgr have defects in adhesion-dependent secondary granule release (Mocsai et al., 1999) and

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Syk-deficient neutrophils exhibit a defect in CD18-induced ROS generation and secondary granule release (Mocsai et al., 2002).

Neutrophil-derived cytotoxic products play an important role in the inflammation of the vessel wall. The local Shwartzman-like reaction (LSR), induced by repeated cytokine injection in the skin, produces histopathologic lesions that are characteristic of thrombohemorrhagic vasculitis (Brozna, 1990) and resemble those observed in SLE in cases where vascular damage occurs in the presence of complement deposition (Danning et al., 1998; Philips et al., 1989; Riemekasten et al., 2002). The LSR is dependent on neutrophils (Argenbright and Barton, 1992; Chang et al., 1993; Stetson and Good, 1951; Zhou et al., 2003). Neutrophil aggregation, their interaction with the endothelium and platelets as well as neutrophil-derived microparticles promote the generation of occlusive thrombi, fibrin accumulation, and hemorrhage (Bouchard and Tracy, 2001; Brozna, 1990; Gasser and Schifferli, 2005).

In this work, we directly tested the contribution of Mac-1, its ligands, effector mechanisms, and signaling pathways to the LSR by using relevant gene mutant mice and intravenous reconstitution of mice with purified neutrophils. While Mac-1-deficient mice showed normal infiltration of neutrophils into the inflammatory site, these mice failed to develop significant hemorrhagic vasculitis, thus separating the neutrophil recruitment versus activation functions of this leukocyte integrin. Mac-1 engagement of complement within the vessel wall signaled activation of Src-family and Syk kinases in a signaling pathway that led to neutrophil elastase release, which in turn was responsible for blood vessel injury. These data delineate an important physiological role for Mac-1-mediated signaling of effector functions in tissue damage associated with thrombohemorrhagic vasculitis.

Results

Mac-1 Is Required for the Formation of Hemorrhagic Lesions

The local Shwartzman reaction (LSR) was elicited by an initial intradermal injection of lipopolysaccharide (LPS) followed 20–24 hr later by a challenge with tumor necrosis factor (TNF) at the same site. 24 hr after TNF injection, hemorrhaging was observed in the intact skin. Microscopic analysis of skin tissue sections revealed significant erythrocyte extravasation, neutrophil accumulation, extravascular fibrin accumulation, and occlusive thrombi containing neutrophils. Mac-1-deficient mice subjected to LSR exhibited no detectable hemorrhage (Figure 1). This was associated with a substantial decrease in the number of thrombus-occluded vessels and reduced fibrin deposition. The fibrin that was present formed a ring within the lumen of intact vessels in contrast to the predominantly extravascular fibrin accumulation observed in wild-type mice. Despite the absence of hemorrhage in Mac-1-deficient mice, neutrophil accumulation in the skin lesions of the mutant mice was essentially equivalent to that of wild-type animals as assessed on tissue sections (Figure 1A). Interestingly, neutrophils adherent to the vessel wall of wild-type animals tended to be flattened against the lumen while those in the Mac-1-deficient animals were round in appearance (Figure 1B, c and d), perhaps reflecting altered adhesion-dependent spreading of the Mac-1-deficient cells. Tissue neutrophil accumulation as assayed by tissue myeloperoxidase (MPO) activity (converted to number of neutrophils via a standard curve) was shown to be comparable in both groups of animals (#neutrophils $\times 10^4$/mg tissue: wt, 2.13 ± 0.17; Mac-1-deficient, 2.02 ± 0.16). Neutrophil accumulation was also similar in wild-type and Mac-1-deficient mice after LPS injection alone or LPS followed by a 4 hr TNF stimulation (data not shown). These data indicate that in the LSR model, the CD18 integrin Mac-1 is not critical for neutrophil recruitment to the inflammatory site but is required for tissue injury leading to the development of thrombohemorrhagic vasculitis.

The LSR was evaluated in LFA-1-deficient mice to assess the relative contribution of this sister CD18 integrin in neutrophil accumulation and tissue injury. LFA-1-deficient mice exhibited only a 20% reduction in hemorrhage (RBC as % of tissue area: wt, 11.7 ± 0.32; LFA-1-deficient, 9.31 ± 0.90, $p < 0.05$, $n = 6$ per group) and a 12% reduction in neutrophil accumulation (data not shown). We conclude that, in contrast to Mac-1, the CD18 integrin LFA-1 does not play a major role in disease pathogenesis in the LSR model.

In vitro studies in macrophages suggest that LPS signaling through CD14 requires Mac-1 (Dobrovolskaia and Vogel, 2002). Ribonuclease protection analysis of total RNA in skin-tissue samples of LPS-injected mice revealed that cytokines upregulated by LPS (IL-1β, IL-1 receptor antagonist, and IL-6) were similar in wild-type and Mac-1-deficient mice. Others, including IFNγ, IL-12 (p35, p40), IL-18, MIF, and IL-1α, did not increase upon LPS treatment in either genotype (Figure 1C, data not shown), and the cytokine profile did not change upon additional challenge with TNF (data not shown). Thus, the phenotype in Mac-1-deficient mice in LSR is likely not the result of altered cytokine production after LPS injection.

Mac-1 on Intravascular Neutrophils Promotes Hemorrhagic Lesions

Mac-1 is present on murine neutrophils, eosinophils, mast cells, monocytes, and tissue macrophages. To examine whether Mac-1 on neutrophils is responsible for hemorrhagic lesions, Mac-1-deficient mice were injected intravenously with purified bone marrow-derived neutrophils harvested from wild-type or Mac-1-deficient mice 2 hr prior to TNF injection in the dorsal skin to induce the LSR. The adoptive transfer of wild-type neutrophils into Mac-1-deficient mice restored the formation of hemorrhagic lesions while the transfer of Mac-1-deficient cells had no effect despite robust neutrophil recruitment to the site of inflammation (Figure 2). The extent of hemorrhage, thrombus formation, and fibrin deposition was essentially the same in Mac-1-deficient mice injected with wild-type neutrophils as seen in normal wild-type animals. In contrast to intravenous transfers, injection of wild-type neutrophils intradermally (at the site of LPS and TNF injection) into Mac-1-deficient animals was not effective in reconstituting disease (data not shown). Therefore, Mac-1 on intravascular

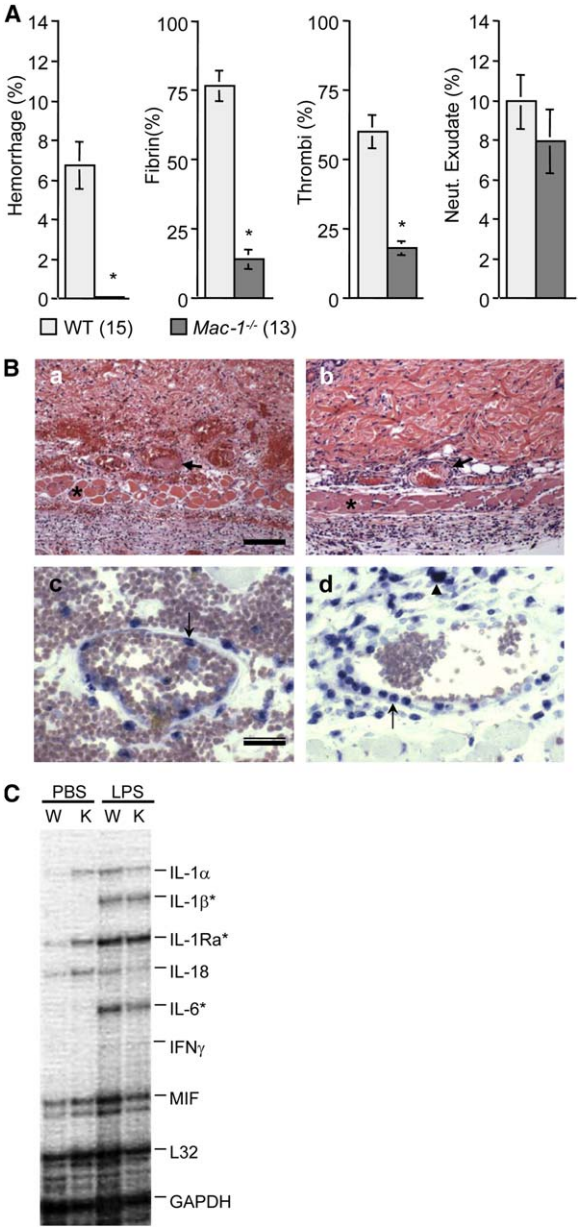

Figure 1. Mac-1-Deficient Mice Fail to Develop Thrombohemorrhagic Vasculitis in the Local Shwartzman-like Reaction Despite Normal Neutrophil Recruitment and Production of Inflammatory Cytokines

(A) Wild-type (wt) and Mac-1-deficient (*Mac-1*$^{-/-}$) mice were subjected to the LSR, and lesions in sections of harvested skin were scored. The percent of tissue area of extravascular RBCs and neutrophils in four low-power fields (40× objective) for hemorrhage and neutrophil (Neut.) exudates, respectively, was determined in wt and *Mac-1*$^{-/-}$ mice. The percent of vessels with fibrin staining outside the vessel wall and the percent of vessels occluded by thrombus were quantitated by scoring all morphologically intact vessels in the skin sample. In parentheses are the numbers of mice of each genotype analyzed to produce the shown averaged data ± SEM, *$p < 0.05$.

(B) Representative skin sections from wild-type (a, c) and *Mac-1*$^{-/-}$ (b, d) mice are shown. (a and b) Hematoxylin and eosin-stained sections show hemorrhaging and thrombosis in wild-type mice with the strongest reaction evident in the deep dermis just above the panniculus carnosus muscle (*). This reaction was not present in *Mac-1*$^{-/-}$ mice. An arrow identifies a blood vessel in each section (c and d). A specific esterase stain revealed neutrophils (small blue cells, arrow) adherent to the vessel wall and in the extravascular space of wild-type and *Mac-1*$^{-/-}$ mice. The larger blue cell (arrowhead in [d]) is a mast cell that also stains positively with the esterase stain and is excluded from the quantitative analysis. Note the flattened appearance of intravascular neutrophils in sections from wild-type (c) compared to the rounded neutrophils in *Mac-1*$^{-/-}$ (d) mice identified by arrows. Scale bar equals 100 μm, double scale bar equals 20 μm.

(C) Total RNA was prepared from skin samples harvested from the LSR site of wild-type and Mac-1-deficient mice. RNA for indicated cytokines and housekeeping genes, L32 and GADPH, was detected in a multiprobe ribonuclease protection assay. A representative autoradiograph of samples from control wild-type (W) and Mac-1-deficient (K) mice after PBS or LPS injection are shown. Cytokines that were upregulated after LPS treatment are indicated by asterisks (*).

neutrophils is an important determinant of the thrombohemorrhagic pathology.

Role for Mac-1 Ligand Complement C3 but Not ICAM-1 in Thrombohemorrhagic Lesions

Mac-1 recognizes several ligands including ICAM-1, complement fragment iC3b (generated by complement C3 activation), GpIb on platelets, and coagulation factors fibrinogen and factor X (Arnaout, 1990; Mayadas and Cullere, 2005). Here, we examined whether Mac-1 ligands ICAM-1 and complement fragment iC3b were required for the development of thrombohemorrhagic lesions by analyzing LSR in mice deficient in ICAM-1 or complement C3 (*C3*$^{-/-}$). ICAM-1-deficient mice had normal neutrophil accumulation with lesion scores that were similar to their wild-type counterparts (Table 1). In contrast, *C3*$^{-/-}$ animals exhibited no hemorrhaging or microthrombi and markedly reduced fibrin deposition despite normal neutrophil accumulation (Table 1). Adherent neutrophils within vessels of *C3*$^{-/-}$ mice exhibited a round morphology, similar to that seen in Mac-1-deficient animals (data not shown). Thus, C3 deficiency recapitulated the phenotype observed in Mac-1-deficient mice.

The distribution of complement C3 deposition in the LSR lesions was examined by immunohistochemistry. Significant C3 deposition was observed along the vessel walls at sites of inflammation (Figure 3). Importantly, the extent of C3 deposition was similar in wild-type and Mac-1-deficient animals. No C3 staining was observed in dorsal skin injected with PBS alone (Figure 3). Together these data suggest that complement C3 is activated and deposits in the vessel wall upon induction of the LSR and that Mac-1-mediated neutrophil interaction with C3 results in vessel injury and leakage leading to thrombohemorrhagic lesions. However, neither C3 nor Mac-1 played a significant role in neutrophil recruitment to the inflammatory sites during the LSR.

Role of NADPH Oxidase-Generated Reactive Oxygen Species and Proteinases in Shwartzman Reaction

Neutrophil degranulation results in the surface translocation and/or release of proteinases (Owen and Campbell, 1999) as well as the production of NADPH oxidase-derived oxygen radicals, both of which are potential mediators of vessel injury. To elucidate the role of various proteinases versus oxygen radicals in the development of vascular lesion during the LSR, we examined mice lacking the serine proteinase neutrophil elastase (NE), metalloproteinases MMP8 or MMP9 alone or both

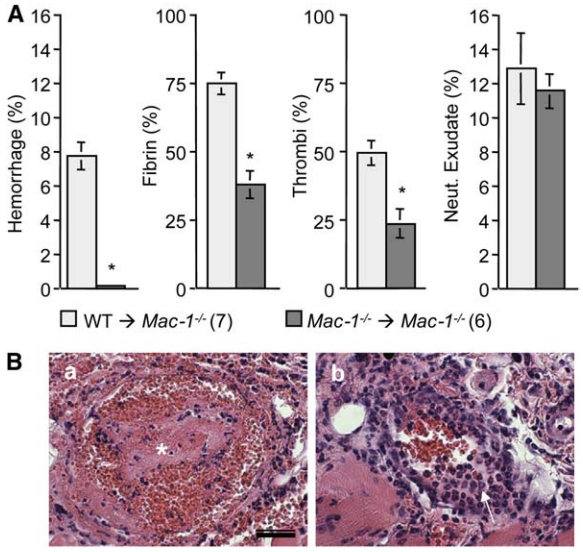

Figure 2. Adoptive Transfer of Wild-Type Neutrophils Restores Thrombohemorrhagic Vasculitis in Mac-1-Deficient Mice

(A) Mac-1-deficient mice primed with LPS were injected once intravenously with 5×10^6 wild-type (wt → *Mac-1*$^{-/-}$) or Mac-1-deficient neutrophils (*Mac-1*$^{-/-}$ → *Mac-1*$^{-/-}$) just prior to intradermal TNF injection. Scoring of skin lesions in tissue sections for parameters of hemorrhaging, thrombi, fibrin, and neutrophil accumulation was conducted as described in Figure 1. Numbers in parentheses are the number of mice used to produce the shown averaged data ± SEM, *$p < 0.05$.

(B) Representative high-magnification images of hematoxylin and eosin-stained sections of Mac-1-deficient mice injected with wild-type (a) or Mac-1-deficient (b) neutrophils are shown. A vessel from Mac-1-deficient animal injected with wild-type neutrophils exhibits hemorrhage and a thrombus (white asterisk) and is distended (a), while a vessel of Mac-1-deficient mouse injected with Mac-1-deficient neutrophils shows significant intralumenal (white arrow) and extravascular neutrophil accumulation but no hemorrhage or thrombus (b). Double scale bar equals 20 μm.

MMP8 and MMP9, or the NADPH-oxidase subunit gp91phox. The LSR was intact in gp91phox-deficient (*gp91*$^{-/-}$) mice, suggesting that reactive oxygen species do not play a major role in the development of lesions or neutrophil accumulation (Table 2A). Interestingly, hemorrhagic and fibrinous lesions were absent in the NE-deficient mice, despite normal neutrophil accumulation (Table 2B), while none of these parameters were altered in mice deficient in either MMP8 or MMP9 alone or in mice doubly deficient in both MMP8 and 9 (Table 2C). Thus, NE is one of the major proteinases that mediate development of thrombohemorrhagic lesions in the LSR without altering cellular recruitment to the inflammatory site, a phenotype also seen in both the *C3*$^{-/-}$ and Mac-1-deficient mice.

Mac-1-Mediated Vessel Injury Requires Neutrophil Elastase

Neutrophil elastase was first identified in neutrophils but is also present in small amounts in monocytes and mast cells (Kawabata et al., 2002; Owen and Campbell, 1999). To test whether elastase in neutrophils alone is responsible for lesion development, the LSR was evaluated in Mac-1-deficient mice injected intravenously with either elastase-deficient or wild-type neutrophils. Mac-1-deficient mice injected with NE-deficient neutrophils exhibited no indices of disease despite significant accumulation of neutrophils at the inflammatory lesion site, while Mac-1 null mice injected with wild-type cells exhibited substantial lesion development (Figures 4A and 4B). These crosscomplementation studies demonstrate that elastase in neutrophils is required for the LSR and that NE is a critical downstream mediator of Mac-1-dependent thrombohemorrhagic lesion development in the LSR.

Next, we evaluated whether Mac-1 deficiency resulted in a decrease in NE release in vivo in response to the LSR. For this, the LSR was induced in an air pouch generated in the dorsal skin of mice, and the air pouch was lavaged to retrieve accumulated neutrophils and fluid. The lavage, as well as the intact neutrophils, were assayed for elastase activity because a portion of the released elastase binds to the surface of neutrophils (Owen et al., 1995). The total number of cells in the lavage of wild-type and Mac-1-deficient mice was similar (wt, $1.56 \pm 0.11 \times 10^6$; Mac-1-deficient, $1.39 \pm 0.04 \times 10^6$). However, elastase release was reduced by approximately 75% in Mac-1-deficient compared to wild-type samples (Figure 4C). These data demonstrate that Mac-1 null neutrophils fail to release NE in vivo during the Shwartzman reaction.

The Src-Family Kinases Are Required for Thrombohemorrhagic Vasculitis: A Dominant Role for Hck

The Src-family kinases in neutrophils, Hck, Fgr, and Lyn play a primary role in integrin-signaling pathways. Deficiency of both Hck and Fgr resulted in a defect in adhesion-mediated activation downstream of β1, β2 (CD18), and β3 integrins, whereas a deficiency in either alone has no detectable phenotype (Lowell et al., 1996). Deficiency in all three of these kinases results in an even more profound defect in integrin signaling, while loss of Lyn alone leads to a hyperresponsive adhesion phenotype—indicative of the dual role of Lyn kinase as both a positive and negative regulator of intracellular

Table 1. C3-Deficient Mice Fail to Develop Thrombohemorrhagic Vasculitis while ICAM-1-Deficient Animals Respond Normally in the Localized Shwartzman Reaction

Grading (%)	Wild-Type (n = 8)	*C3*$^{-/-}$ (n = 10)	Wild-Type (n = 4)	*ICAM-1*$^{-/-}$ (n = 4)
Hemorrhage	5.98 ± 2.22	0.00 ± 0.00*	10.86 ± 2.19	9.78 ± 2.35
Fibrin	77.38 ± 2.57	14.03 ± 1.78*	79.75 ± 4.14	81.70 ± 9.09
Thrombi	78.59 ± 2.92	20.44 ± 2.61*	62.32 ± 3.36	60.92 ± 9.90
Neut. Exudate	11.06 ± 2.31	10.37 ± 2.03	12.35 ± 1.32	13.48 ± 1.25

Skin sections from LSR lesions in wild-type, C3-deficient (*C3*$^{-/-}$), or ICAM-1-deficient (*ICAM-1*$^{-/-}$) mice were prepared and scored as described in Figure 1. n = number of mice of each genotype analyzed to produce the averaged data, *$p < 0.05$ compared to wild-type cohorts.

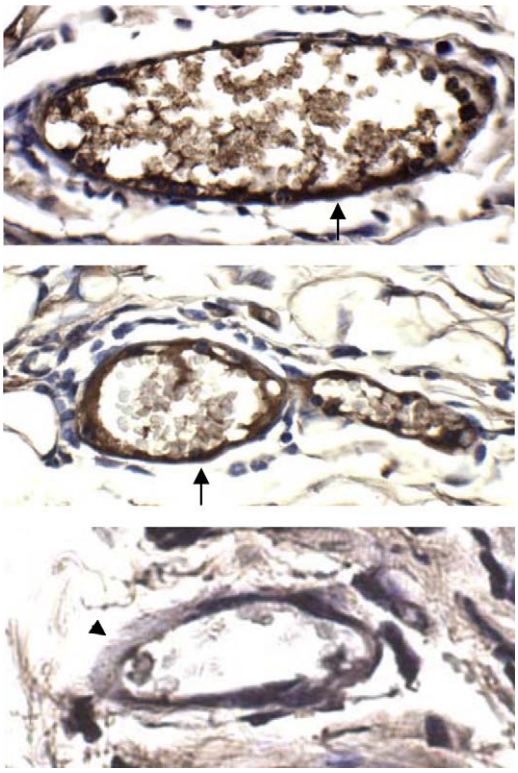

Figure 3. Complement C3 Is Deposited along Vessel Walls during Experimental Thrombohemorrhagic Vasculitis

Skin-tissue sections from wt (top) or Mac-1-deficient (middle) mice subjected to the LSR and wt mice injected with PBS alone (bottom) were stained with polyclonal anti-C3 antibody. C3 staining is observed along a vessel (arrow) from a wild-type (top) and Mac-1-deficient (middle) animal subjected to LSR, while no C3 staining is observed in a vessel (arrowhead) of a wild-type mouse injected with PBS alone (bottom). Complement C3-deficient mice subjected to LSR showed no staining (not shown), demonstrating the specificity of the C3 antibody.

signaling (Mocsai et al., 2002; Pereira and Lowell, 2003). To examine whether Src-family kinases are critical for Mac-1-mediated signaling in vivo, we analyzed mice deficient in Hck, Fgr, or Lyn alone as well as mice lacking all three kinases in the LSR. *Fgr*$^{-/-}$ mice developed hemorrhagic lesions that were partially reduced compared to wild-type animals while Lyn deficiency resulted in a further modest reduction in disease indices (Figure 5A). In both these kinase mutant strains, all animals showed clear evidence of some hemorrhage after TNF injection. In contrast, 73% of *Hck*$^{-/-}$ mice were completely free of extravascular hemorrhage and fibrin deposition, with the remaining animals displaying variable amounts of disease formation. Triple src-deficient mice exhibited no hemorrhage and significantly reduced fibrin deposition in response to the LSR (Figure 5A). These data indicate that the Src-family kinases in myeloid leukocytes have partially redundant roles in initiating thrombohemorrhagic vasculitis, with the Hck kinase playing the most important function. Loss of Hck alone or deficiency of all three kinases produced a profound impairment in hemorrhage and fibrin formation in the LSR model, without affecting neutrophil migration (Figure 5A), a phenotype very similar to that of the C3-, Mac-1-, or neutrophil elastase-deficient mice.

To evaluate whether the phenotypes observed in the single and triple src mutant mice could be attributed to the role of Src-family proteins specifically in neutrophils, Mac-1-deficient mice were injected with neutrophils from either *Hck*$^{-/-}$, *Fgr*$^{-/-}$, or triple src mutant mice and examined in the LSR. Mac-1-deficient mice injected with *Hck*$^{-/-}$ neutrophils prior to initiation of the LSR showed significantly reduced hemorrhage and modestly reduced fibrin deposition compared to injection of wild-type cells (Figures 5B and 5C). Adoptive transfer of triple src mutant neutrophils into Mac-1-deficient mice failed to reconstitute disease formation; the Mac-1-deficient recipients in these experiments showed no evidence of extravascular hemorrhage or fibrin deposition. Thus, reconstitution of Mac-1 mutant mice with either *Hck*$^{-/-}$ or triple src-deficient neutrophils produces the same phenotypic response in the LSR as seen in the original kinase mutant animals, indicating that loss of these kinases in neutrophils alone is responsible for the amelioration of inflammatory disease in this model. In contrast, Mac-1-deficient mice that were injected with *Fgr*$^{-/-}$ neutrophils showed nearly normal levels of hemorrhage (Hemorrhage %: wt neutrophils, 12.58 ± 0.39; *Fgr*$^{-/-}$ neutrophils, 9.81 ± 1.11, $p = 0.40$), which differed from the moderate reduction in hemorrhage seen in the *Fgr*$^{-/-}$ mice alone. Thus, the loss of Fgr from cells other than neutrophils likely contributed to the partially reduced response to the LSR observed in *Fgr*$^{-/-}$ mice (Figure 5A).

To further validate that loss of Src-family kinases in neutrophils alone is capable of blocking disease activity in the LSR and that Mac-1 is upstream of these kinases, we attempted to restore disease to triple src mutant mice by injection of wild-type or Mac-1-deficient neutrophils. As expected, triple src mutant mice injected with wild-type neutrophils displayed normal levels of hemorrhage and fibrin deposition after initiation of the LSR, while triple src mutant mice injected with Mac-1-deficient cells remained free of disease (Figures 5B and 5C). These crosscomplementation experiments confirm that Src-family kinases in neutrophils are required for thrombohemorrhagic vasculitis.

Syk Is Required for Mac-1-Dependent Vasculopathy

The described role for the Syk in outside-in integrin signaling in neutrophils in vitro (Mocsai et al., 2002) prompted us to determine whether this kinase is also required for Mac-1-dependent thrombohemorrhagic vasculitis in vivo. To examine this, chimeric mice were generated by injecting fetal liver cells from *Syk*$^{-/-}$ mice into lethally irradiated wild-type recipients, allowing us to circumvent the perinatal lethality caused by the *Syk* mutation (Turner et al., 2000). Controls for these animals were irradiated wild-type mice injected with fetal liver cells from wild-type or *Syk*$^{+/-}$ animals. Surprisingly, even wild-type mice reconstituted with wild-type cells after lethal irradiation failed to develop reproducible evidence of thrombohemorrhagic vasculitis in the LSR (data not shown), precluding the examination of intact chimeras in these experiments. Therefore, the role of Syk kinase in neutrophils in the LSR was examined by attempting to restore disease in Mac-1-deficient mice with neutrophils from wild-type and *Syk*$^{-/-}$ fetal liver chimeric mice. Mac-1-deficient mice injected with neutrophils from wild-type or *Syk*$^{+/-}$ fetal liver chimeric mice

Table 2. Neutrophil Elastase-Deficient Mice Fail to Develop Thrombohemorrhagic Vasculitis in the LSR, while Mice Deficient in NADPH Oxidase Function or Lacking MMP8 and MMP9 Respond Normally

(A) Mice Lacking the gp91phox Subunit of the NADPH Oxidase ($gp91^{-/-}$)		
Grading (%)	Wild-Type (n = 10)	$gp91^{-/-}$ (n = 10)
Hemorrhage	6.28 ± 0.74	4.64 ± 1.87
Fibrin	73.03 ± 6.13	61.13 ± 20.50
Thrombi	57.62 ± 5.56	52.35 ± 8.93
Neut. Exudate	9.84 ± 1.13	7.15 ± 1.29

(B) Mice Lacking the Primary Granule Proteinase Neutrophil Elastase ($NE^{-/-}$)		
Grading (%)	Wild-Type (n = 6)	$NE^{-/-}$ (n = 5)
Hemorrhage	8.91 ± 1.03	0.02 ± 0.02*
Fibrin	83.40 ± 0.32	34.50 ± 12.18*
Thrombi	54.31 ± 9.96	14.34 ± 1.52*
Neut. Exudate	14.78 ± 2.23	10.91 ± 1.39

(C) Mice Lacking the Primary Granule Proteinases MMP8 and/or MMP9 (Single $MMP8^{-/-}$ or $MMP9^{-/-}$ Mutants or Double $MMP8/MMP9^{-/-}$ Mutants)				
Grading (%)	Wild-Type (n = 5)	$MMP9^{-/-}$ (n = 3)	Wild-Type (n = 8)	$MMP8^{-/-}$ (n = 6)
Hemorrhage	12.44 ± 3.54	14.47 ± 6.91	9.66 ± 1.04	10.59 ± 2.34
Fibrin	75.30 ± 3.36	74.90 ± 14.13	81.47 ± 2.41	76.65 ± 3.35
Thrombi	55.50 ± 9.18	61.30 ± 21.55	71.17 ± 2.84	68.25 ± 4.80
Neut. Exudate	13.63 ± 1.78	17.06 ± 0.33	11.33 ± 1.38	9.42 ± 2.22
Grading (%)	Wild-Type (n = 4)	$MMP8/MMP9^{-/-}$ (n = 3)		
Hemorrhage	9.46 ± 1.66	12.12 ± 1.71		
Fibrin	77.65 ± 3.38	77.56 ± 4.52		
Thrombi	66.01 ± 4.25	74.14 ± 5.96		
Neut. Exudate	10.96 ± 2.40	9.55 ± 1.65		

LSR lesions in wild-type and indicated deficient mice were assessed for hemorrhage, thrombi, fibrin deposition, and neutrophil accumulation as described in Figure 1. n = number of mice of each genotype analyzed to produce the averaged data, *$p < 0.05$ compared to wild-type cohorts.

developed hemorrhage and fibrin deposition in the LSR, while Mac-1-deficient mice receiving $Syk^{-/-}$ cells failed to show evidence of disease (Table 3). As seen in the $C3^{-/-}$, Mac-1-deficient, NE-deficient, and the *Hck,Fgr,* $Lyn^{-/-}$ triple knockout mice, $Syk^{-/-}$ neutrophils were recruited normally into the inflammatory site, indicating that this kinase is not involved in neutrophil migration, as previously reported in a thioglycollate-induced peritonitis model (Mocsai et al., 2002). These results directly implicate Syk kinase in neutrophils in the development of Mac-1-mediated thrombohemorrhagic vasculitis at a step involving neutrophil activation and elastase release, without altering neutrophil recruitment.

Mac-1 Adhesion-Dependent Release of Elastase In Vitro Requires Complement C3 and Hck Kinase

Our in vivo results suggest that Mac-1-mediated adhesion to complement results in elastase release that is largely dependent on the Src-family kinase Hck. Previous reports have shown that adhesion of activated neutrophils to surfaces coated with integrin ligands (e.g., fibrinogen or collagen) results in the exocytosis of primary (azurophilic) and secondary (specific) granules (Mocsai et al., 1999, 2002). We designed an in vitro assay to assess the role of Src kinases in Mac-1-mediated adhesion and release of elastase (present in primary granules) to complement-coated surfaces after TNF stimulation. Since biomaterials such as tissue-culture plates are well described to activate complement C3 resulting in deposition of C3b and iC3b (Andersson et al., 2002; Chenoweth et al., 1981), this assay presents high-density Mac-1 ligands to the leukocyte, similar to the situation of vascular deposition of C3 components in the LSR. The adhesion of TNF-stimulated wild-type neutrophils to plates coated with freshly prepared mouse serum was dependent on Mac-1 and complement, as shown by the fact that both Mac-1-deficient neutrophils and $C3^{-/-}$ serum failed to support significant neutrophil adhesion (Figure 6A). To measure the adhesion-dependent release of neutrophil elastase, we used a continuous spectroscopic assay containing a fluorogenic substrate specific for neutrophil elastase (Sklar et al., 1982). Adhesion of wild-type neutrophils to complement-coated plates resulted in significant elastase release, whereas Mac-1-deficient neutrophils or wild-type neutrophils adherent to plates coated with $C3^{-/-}$ serum exhibited significantly less elastase release (Figure 6B). Similar to the adhesion assay, primary granule release was very low in Mac-1-deficient cells plated on $C3^{-/-}$ serum. Thus, TNF-induced Mac-1 interaction with complement iC3b is responsible for the majority (greater than 70%) of elastase release observed in this assay.

The role of Src-family kinases in Mac-1 and C3 adhesion-mediated elastase release was evaluated by assessing degranulation in neutrophils deficient in the Hck, Fgr, or Lyn kinases alone or all three src kinases. As shown in Figure 6C, both the $Hck^{-/-}$ and triple src mutant cells manifested a large reduction in degranulation after adhesion to wild-type serum-coated surfaces. In contrast, $Fgr^{-/-}$ and $Lyn^{-/-}$ cells showed no significant decrease in elastase release in this assay. These in vitro elastase results correlate well with the in vivo observations in the LSR and suggest that the Hck kinase is the dominant neutrophil Src-family kinase involved

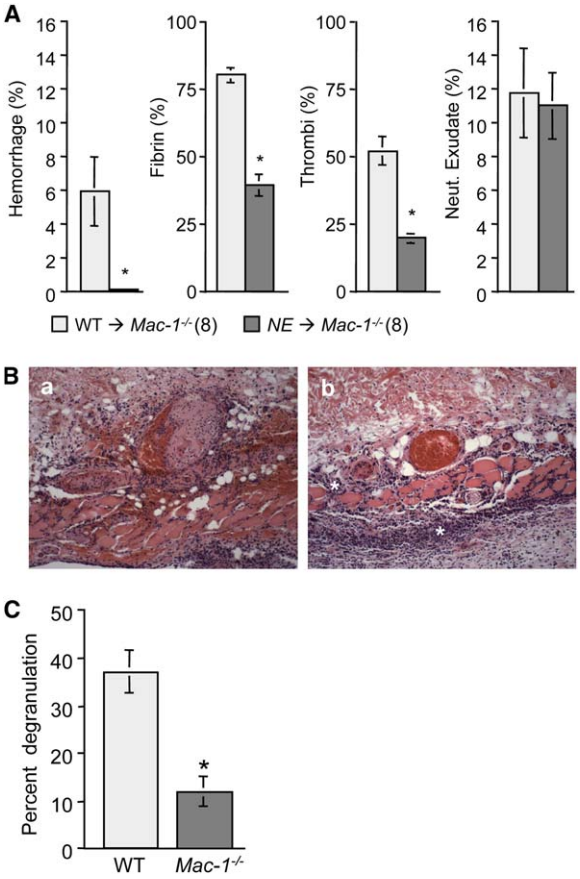

Figure 4. Adoptive Transfer of Elastase-Deficient Neutrophils Fails to Restore Thrombohemorrhagic Vasculitis to Mac-1-Deficient Mice

(A) Mac-1-deficient (*Mac-1*$^{-/-}$) mice primed with LPS were injected once intravenously with 5 × 10^6 wild-type (wt → *Mac-1*$^{-/-}$) or neutrophil elastase-deficient (*NE*$^{-/-}$ → *Mac-1*$^{-/-}$) cells just prior to intradermal TNF injection, and lesions were scored as outlined in Figure 1, Numbers in parentheses are the number of mice used to produce the shown averaged data ± SEM, *p < 0.05.

(B) Representative sections of Mac-1-deficient mice injected with wild-type (a) or *NE*$^{-/-}$ (b) neutrophils are shown. Mac-1-deficient mice injected with wt cells exhibited significant hemorrhage, thrombus formation, and neutrophil infiltration (a), while those injected with *NE*$^{-/-}$ cells (b) displayed intense neutrophil infiltration at the TNF injection site (white asterisk) without evidence of hemorrhage or thrombus formation.

(C) Skin air pouches were generated in Mac-1-deficient (*Mac-1*$^{-/-}$) and wild-type (wt) mice and the LSR was induced in this site. The pouches were lavaged, and intact neutrophils and lavage fluid were assayed for neutrophil elastase activity. The percent degranulation was calculated by dividing the elastase activity by the total cellular elastase activity measured after lysis of cells in the sample. n = 4 for each genotype. Data shown are average ± SD, *p < 0.05.

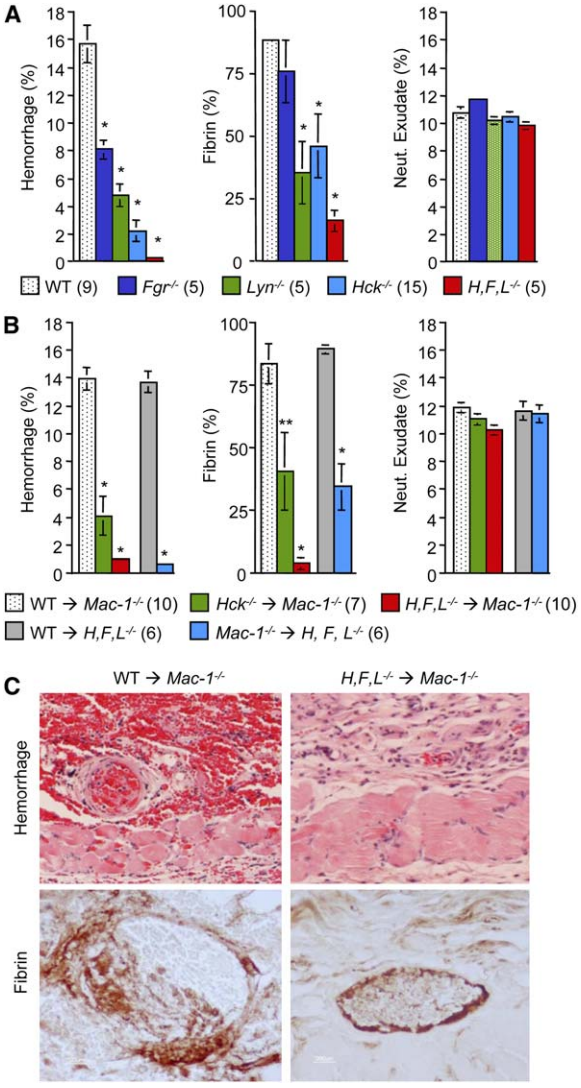

Figure 5. Lack of Src-Family Kinases in Neutrophils Renders Mice Resistant to Experimental Thrombohemorrhagic Vasculitis

(A) Wild-type, *Fgr*$^{-/-}$, *Lyn*$^{-/-}$, *Hck*$^{-/-}$, or triple src mutant (*H,F,L*$^{-/-}$) mice were subjected to the LSR, and scoring of tissue sections for percent hemorrhage, extravascular fibrin deposition, and neutrophil exudates was performed as in Figure 1.

(B) Mac-1-deficient mice primed with LPS were injected once intravenously with 5 × 10^6 wild-type (wt → *Mac-1*$^{-/-}$), single mutant *hck*$^{-/-}$ (*Hck*$^{-/-}$ → *Mac1*$^{-/-}$), or triple src mutant (*H,F,L*$^{-/-}$ → *Mac-1*$^{-/-}$) neutrophils just prior to intradermal TNF injection, and lesions were scored as outlined in Figure 1. In addition, *H,F,L*$^{-/-}$ mutant mice served as recipients for injection of either wild-type (wt → *H,F,L*$^{-/-}$) or Mac-1-deficient (*Mac-1*$^{-/-}$ → *H,F,L*$^{-/-}$) cells, after which mice were analyzed in the LSR as above. Numbers in parentheses indicate the number of animals of each genotype analyzed to produce the shown averaged data ± SEM. *p < 0.005, **p < 0.05 in comparison to wild-type.

(C) Photomicrographs show representative hematoxylin and eosin (hemorrhage) or fibrin-stained skin sections from Mac-1-deficient mice injected with either wild-type cells (wt → *Mac-1*$^{-/-}$) or triple knockout cells (*H,F,L*$^{-/-}$ → *Mac-1*$^{-/-}$).

in signaling primary granule release after adhesion to surface bound complement.

Discussion

Our studies establish a key role for Mac-1 in the destruction of vascular integrity associated with a model of thrombohemorrhagic vasculitis. These data are consistent with previous reports showing inhibition of LSR lesions after treatment with CD11b blocking antibody (Argenbright and Barton, 1992; Chang et al., 1993). The near absence of tissue injury in Mac-1-deficient mice was not the result of changes in cytokines induced during the LSR (IL-6, IL-1β, IL-1ra), nor was it attributable to reduced neutrophil accumulation in the skin. Thus the LSR model allows us to separate the function of integrin

Table 3. Adoptive Transfer of Syk-Deficient Neutrophils Fails to Restore Normal Disease Activity in Mac-1-Deficient Mice Undergoing LSR

Recipient	*Mac-1*$^{-/-}$ (n = 8)	*Mac-1*$^{-/-}$ (n = 8)
Donor	Wild-Type or *Syk*$^{+/-}$	*Syk*$^{-/-}$
Hemorrhage (%)	12.55 ± 0.24	0.38 ± 0.10*
Fibrin (%)	92.40 ± 3.16	6.87 ± 0.49*
Neut. Exudate (%)	12.97 ± 0.55	9.06 ± 0.40

Mac-1-deficient mice (*Mac-1*$^{-/-}$) primed with LPS were injected once intravenously with 5 × 10^6 wild-type/*Syk*$^{+/-}$ or Syk-deficient (*Syk*$^{-/-}$) neutrophils just prior to intradermal TNF injection. Neutrophils were prepared from fetal liver chimeric mice that had previously been established with wild-type/*Syk*$^{+/-}$ or *Syk*$^{-/-}$ cells. Scoring of skin lesions in the adoptively transferred *Mac-1*$^{-/-}$ tissue sections for parameters of hemorrhaging, fibrin deposition, and neutrophil accumulation was conducted as described in Figure 1. n = number of mice analyzed to produce the averaged data, *p < 0.05 compared to wild-type/*Syk*$^{+/-}$ cohorts.

(i.e., Mac-1)-dependent inflammatory cell recruitment from integrin-mediated cellular activation in an in vivo inflammatory disease. In the vast majority of experimental inflammation models, the presence of tissue injury usually correlates with inflammatory (neutrophil) cell infiltrate into the lesion sites. However, in the LSR model, this is not the case—all of the mutant mice we have examined displayed normal neutrophil recruitment into the disease site independent of vascular injury and hemorrhage. We have taken advantage of these aspects of the LSR model to study the upstream ligands, downstream signaling molecules, and effector systems of neutrophil Mac-1, thereby allowing us to analyze the contribution of this integrin to cellular activation and resulting inflammatory tissue injury in vivo. Our studies indicate that interaction of Mac-1 on neutrophils with complement iC3b deposits within the vessel wall leads to an intracellular signaling pathway involving Src-family and Syk kinases, causing degranulation and vascular injury that is dependent on release of neutrophil elastase (Figure 7).

Mice deficient in complement C3, which therefore are unable to form the Mac-1 ligand iC3b, phenocopied Mac-1-deficient mice in all aspects of the LSR, consistent with a previous report suggesting suppression of the LSR after complement depletion (Fong and Good, 1971). This suggests that the interaction of Mac-1 on neutrophils with complement C3 that is deposited along the vascular walls at the site of TNF injection promotes neutrophil activation leading to tissue damage. Complement may be on the endothelium (Marks et al., 1989) and/or in the subendothelial matrix, which can fix iC3b upon its exposure after cytokine-induced endothelial cell retraction (Hindmarsh and Marks, 1998). Our finding that restoration of disease in Mac-1-deficient mice required the intravenous delivery of wild-type neutrophils suggests that the interaction of Mac-1 on circulating neutrophils with fixed complement must occur within the vascular lumen for significant vessel damage to occur (Figure 7). Previous reports have suggested that complement deposition in the vasculature is consistently associated with vasculitides (Chen et al., 2002; Danning et al., 1998; Prins et al., 1996). Mac-1 interaction with complement C3bi could drive neutrophil degranulation at the vessel wall via a process often referred to as

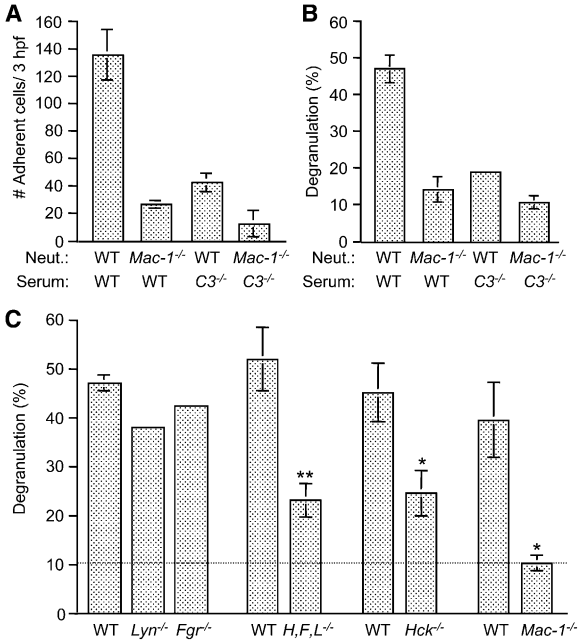

Figure 6. Adhesion of Neutrophils to Complement-Coated Surfaces Leads to Elastase Release in Wild-Type but Not Mac-1- or Hck Kinase-Deficient Cells

(A) Analysis of adhesion of TNF-stimulated wild-type or Mac-1-deficient (*Mac-1*$^{-/-}$) neutrophils to wild-type or C3-deficient (*C3*$^{-/-}$) fresh mouse serum-coated plates. TNF-stimulated neutrophils were incubated with plates for 40 min, plates were washed, and the number of attached neutrophils in three high-power fields was counted microscopically. Data shown are the average ± SD of two independent experiments.

(B) The release of the azurophilic granule marker neutrophil elastase was assessed after plating of TNF-activated wild-type or *Mac-1*$^{-/-}$ neutrophils on fresh mouse serum-coated dishes. The data are plotted as % degranulation, which represents the amount of neutrophil elastase released from 2.5 × 10^3 neutrophils per well, divided by the total cellular elastase activity (determined by lysis of cells in 0.02% Triton X-100) after 60 min of adhesion to the indicated serum-coated surface. Data shown are the average ± SD of two independent experiments.

(C) Release of neutrophil elastase from wild-type, *Mac-1*$^{-/-}$, *Hck*$^{-/-}$, *Fgr*$^{-/-}$, *Lyn*$^{-/-}$, or triple src mutant (*H,F,L*$^{-/-}$) neutrophils was conducted as described above. Data shown are the average ± SD. n = 4 to 8 independent experiments for each group except for the first (wt, *Fgr*$^{-/-}$, and *Lyn*$^{-/-}$), which is n = 2.

*p < 0.005, **p < 0.05 in comparison to respective wt.

"frustrated phagocytosis," where phagocytes may form a tight seal around the complement deposits, creating a compartment into which secreted proteinases can be concentrated and protected from serum proteinase inhibitors, thus maximizing endothelial cell injury (Heiple et al., 1990; Wright and Silverstein, 1984).

Previous work has shown that treatment with CD18 antibody results in the reduction in LSR-induced hemorrhagic legions in rabbits (Argenbright and Barton, 1992), although the effect of CD18 blockade on neutrophil accumulation was not reported. Neutrophil recruitment in the LSR was essentially normal in Mac-1-deficient, LFA-1-deficient, and ICAM-1-deficient mice. Thus, it is possible that a combination of LFA-1 and Mac-1 contributes to neutrophil recruitment in the LSR model. Indeed, only an ablation of both Mac-1 and LFA-1 significantly reduced neutrophil accumulation in

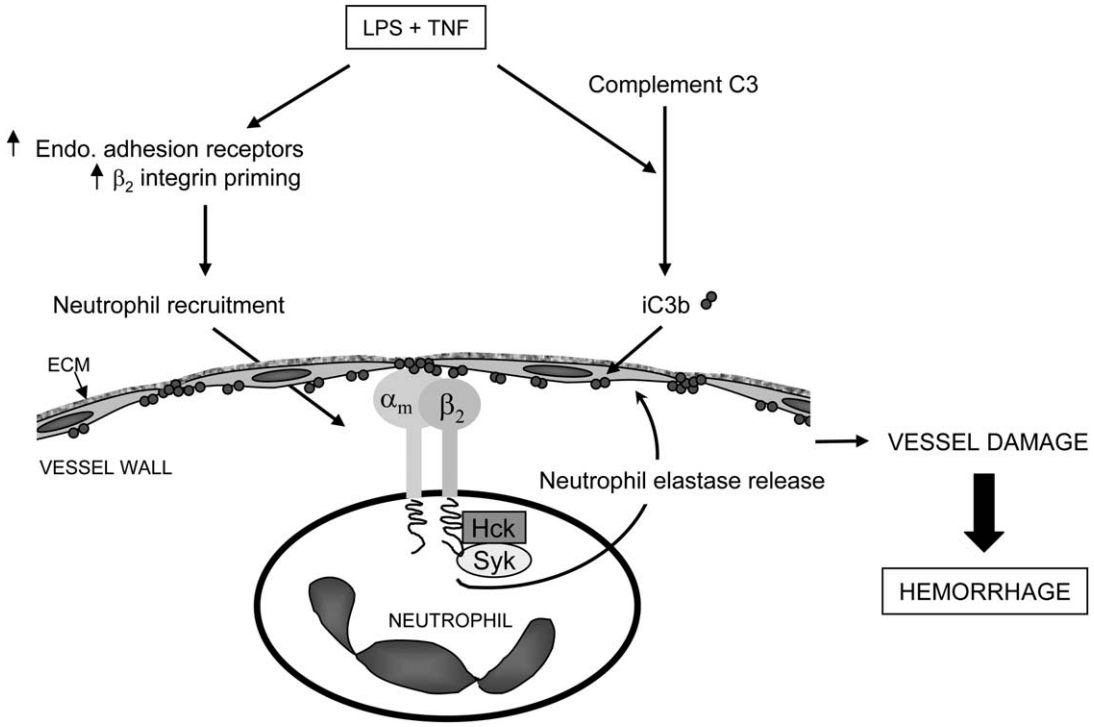

Figure 7. Model of Mac-1-Mediated Inflammation-Induced Vascular Injury and Thrombosis in the Local Shwartzman Reaction

Activation of the alternative complement pathway occurs continuously in vivo through the C3 "tick-over" pathway. LPS, the "preparative agent" in the Shwartzman reaction, significantly increases C3 synthesis (Colten et al., 1986; Thorbecke et al., 1965), and TNF promotes synthesis and release of complement components C3 and factor B by endothelial cells, thus leading to further opportunities for local activation of complement (Kawakami et al., 1997). TNF promotes endothelial cell retraction, with subsequent exposure of the subendothelial extracellular matrix (ECM), which is a target for C3b/iC3b fixation (Hindmarsh and Marks, 1998). LPS and TNF upregulate adhesion receptors required for neutrophil accumulation. Neutrophils are recruited (in a fashion independent of Mac-1 and complement), allowing Mac-1 ($\alpha_m\beta_2$) on the cell surface to bind the iC3b deposited within the vessel wall. This triggers activation of Src-family and Syk kinases, leading to an intracellular signaling cascade that results in flattening of the neutrophil against the vessel wall and stimulation of localized degranulation, leading to neutrophil elastase release. The elastase promotes vascular injury contributing to tissue hemorrhage.

a peritonitis model (Lu et al., 1997). Furthermore, since ICAM-1$^{-/-}$ mice were unaffected, the CD18 integrins may recognize another endothelial adhesion ligand such as ICAM-2 or a combination of ICAM-1 and ICAM-2 to mediate neutrophil influx. On the other hand, as anti-CD18 mAb injection leads to only a modest reduction (45%) in cellular accumulation in an LPS-induced hemorrhage model (Zhou et al., 2003), it is possible that other integrins (β1 or β3) play a more important role in the adhesive event leading to transmigration of neutrophils out of the vessels. The chemoattractants responsible for neutrophil recruitment in the LSR are unknown. Since C3 deficiency had no effect on neutrophil accumulation, the C3 cleavage product C3a, as well as C5a (which are powerful neutrophil chemoattractants), are not seminal to neutrophil accumulation in the LSR. Further investigation will be required to dissect the adhesion receptors and chemokine/chemoattractant pathways that are essential to neutrophil accumulation in this disease model.

Proteinases and oxygen radical species released from accumulated neutrophils are potential effectors of vessel injury and thrombosis. Neutrophil-derived oxygen radicals may promote tissue injury through oxidation of tryptophan residues or production of bityrosine in matrix molecules (Riedle and Kerjaschki, 1997), by indirectly activating latent collagenase and gelatinase, and/or neutralization of proteinase inhibitors such as α1 anti-trypsin and secretory leukoprotease inhibitor (Kawabata et al., 2002). Neutrophils may also directly modulate blood coagulation through the release of oxygen radicals and proteinases (Bouchard and Tracy, 2001). Our studies demonstrated that NADPH oxidase-derived reactive oxygen species were not significant effectors of thrombohemorrhagic vasculopathy in the LSR. Neutrophils store an arsenal of proteinases in primary (elastase, cathepsin G, and PR3), secondary (MMP8, 80% uPA), and tertiary (MMP9, 20% uPA) granules (Owen and Campbell, 1999) that can promote vessel damage and thrombosis in the LSR (Movat and Wasi, 1985). Despite a demonstrated role for MMP9 in mice in several experimentally induced inflammatory or autoimmune pathologies (Liu et al., 1998; Opdenakker et al., 2001; Shapiro, 1998) and MMP8 in the inflammatory response induced by carcinogens (Balbin et al., 2003), these two metalloproteinases played no detectable role in tissue injury in our model. In contrast, neutrophil elastase, which degrades diverse substrates in vitro including matrix macromolecules, plasma proteins, proinflammatory mediators, and adhesion receptors, was required for lesion development, demonstrating that release of this enzyme is the primary cause of tissue injury in the LSR. Moreover, our crosscomplementation experiments demonstrated that elastase specifically in

neutrophils (versus other leukocytes) was involved. The importance specifically of elastase versus other MMPs for the development of vasculopathy suggests that despite the large number of proteinases that are frequently coexpressed and their broad substrate specificity in vitro, each enzyme may be restricted to a very specific function in vivo. Our in vivo analyses suggested that Mac-1-mediated release of elastase is important in the development of disease. While we believe that the development of hemorrhagic lesions is due mainly to Mac-1-dependent signaling leading to elastase release, it is also possible that Mac-1 contributes to disease pathogenesis by directly retaining elastase on the neutrophil surface (Cai and Wright, 1996) and thereby concentrating its proteolytic activity (Owen and Campbell, 1999).

The downstream signaling reactions after leukocyte integrin engagement have been well studied with cell-based assay methods (Berton et al., 2005; Lowell, 2004). Engagement of Mac-1 leads to activation of Src-family and Syk kinases, which in turn signal to downstream molecules such as SLP-76 and the Vav-family exchange factors. Activation of this signaling pathway leads to degranulation and respiratory burst in neutrophils. The critical role of these signaling molecules has been defined by examination of neutrophil functional responses in vitro by means of cells isolated from the respective knockout mice (Gakidis et al., 2004; Lowell, 2004; Newbrough et al., 2003). However, there have been very few studies of the functional roles of these signaling molecules in in vivo disease models that reflect integrin function. Mice lacking both Hck and Fgr have reduced mortality and tissue damage in a systemic endotoxin model, which correlates with reduced neutrophil accumulation in the liver parenchyma (Lowell and Berton, 1998). Similarly, $Fgr^{-/-}$ mice have reduced eosinophil accumulation during allergic pneumonitis (Vicentini et al., 2002). There are no reports of the contribution of Syk kinase in an in vivo disease model. Our results illuminate the central role of this kinase and the overlapping roles of Src-family kinases in a Mac-1-dependent inflammatory injury model (Figure 7), thus validating the physiologic significance of prior in vitro cell-based experiments.

Previous studies indicate a largely redundant role for the Src-family kinases downstream of integrin ligation. This is especially true for Hck versus Fgr—no unique functional role for these kinases has been defined in cell-based experiments. Thus, the dominant role for the Hck kinase in the primary granule release response after complement binding by neutrophils, which correlated with the reduced disease in the LSR observed in the $Hck^{-/-}$ mice, is one of the few examples available demonstrating the functional importance of a specific Src-family kinase in leukocyte function. Hck has been localized to primary granules and has been shown to translocate to the phagosomal membrane after serum-opsonized zymosan phagocytosis (Mohn et al., 1995), consistent with a major functional role in primary granule mobilization in neutrophils.

By using a genetic approach and adoptive neutrophil transfer methods, we have defined a signaling pathway beginning with Mac-1 recognition of fixed complement, leading to activation of Src-family and Syk kinases that in turn stimulate neutrophil activation in which release of a specific proteinase (NE) is responsible for the hemorrhagic vasculitis in the Shwartzmann reaction (Figure 7). Integrin signaling leading to activation of neutrophil effector function has been postulated to contribute to tissue injury during inflammation. However, in the majority of disease models, it is difficult to validate a role for integrins in leukocyte activation independent of their role in cellular recruitment. The mechanism of the pathophysiology in the LSR has allowed us to separate these integrin functions and demonstrate a clear role for integrin signaling through nonreceptor tyrosine kinases to leukocyte activation in an in vivo disease model. The obvious implication of these studies is that inhibitors directed against the signaling molecules downstream of Mac-1 will provide novel therapeutic approaches to the treatment of vasculitis and potentially other inflammatory disease states.

Experimental Procedures

Reagents and Antibodies

Recombinant murine TNF was from R&D systems (Minneapolis, MN). *Escherichia coli* LPS 055:B5 was from Sigma (St. Louis, MO). These studies were conducted with LPS that had high endotoxin activity (1,500,000 endotoxin units/mg) and low protein contamination (1.4%) according to manufacturer's certificate of analysis. Rabbit anti-human fibrinogen and rabbit anti-human C3 complement were from DAKO (Carpinteria, CA).

Mice

Gene-deleted mice backcrossed to C57Bl/6 are denoted as B6 with a F# designating the number of generations the animals were backcrossed to C57Bl/6. The gene nomenclature in mice is given in parentheses. $gp91phox^{-/-}$ (*Cybb*) mice B6F12 and LFA-1-deficient (*Itgal*) mice B6F12 were purchased from Jackson Laboratory (Bar Harbor, ME). Mac-1-deficient (*Itgam*) mice (Coxon et al., 1996) are B6F9 and were bred and maintained in the Viral Antigen Free facility at the Longwood Medical Research Center (LMRC) animal housing facility at Harvard Medical School. *ICAM-1*-deficient (*Icam1*) mice (Xu et al., 1994) B6F7 were bred and maintained at the CBR Institute of Biomedical Research, Harvard Medical School (Boston, MA). Complement C3-deficient (C3) (Wessels et al., 1995) mice B6F10 were generously provided by Dr. Michael C. Carroll (CBR Institute of Biomedical Research). Neutrophil elastase-deficient (*Ela2*) mice (Belaaouaj et al., 1998) were B6F10. $Hck^{-/-}$ *(Hck),* $Fgr^{-/-}$ (*Fgr*), $Lyn^{-/-}$ (*Lyn*), and triple src-deficient *Hck,Fgr,*$Lyn^{-/-}$ mice were all B6F15 and maintained in the SPF facility at UCSF. $Syk^{-/-}$ (*Syk*) B6F6 heterozygous mice were used for production of $Syk^{-/-}$ fetal liver stem cells used for reconstitution of lethally irradiated B6 recipients, to generate $Syk^{-/-}$ fetal liver chimeras as described (Mocsai et al., 2002). Age-matched wild-type C57Bl/6 mice were used for all the aforementioned C57Bl/6 gene-deleted animals and were bred in the LMRC facility or purchased from Jackson Laboratory. For $Syk^{-/-}$ fetal liver chimeras, sibling fetal liver cells from either wild-type or $Syk^{+/-}$ mice were used to generate control chimeras. $Syk^{-/-}$ versus wild-type or heterozygous fetal animals were determined by visual inspection (the $Syk^{-/-}$ fetus has an obvious bleeding/edema phenotype), and fetal liver was used immediately for transfer to irradiated recipients without separately identifying wild-type versus $Syk^{+/-}$ cells. Neutrophil elastase-deficient mice and mice deficient in MMP8 (*Mmp8*) (C57Bl/6/129Sv mixed strain) (Balbin et al., 2003) or MMP9 (*Mmp9*) (pure 129Sv) (Vu et al., 1998) or mice deficient in both MMP8 and MMP9 were bred in the Viral Antigen Free facility at Harvard School of Public Health animal housing facility and maintained in the LMRC facility. Age-matched wild-type controls for MMP8- and MMP9-deficient mice were C57/129 strain (B6129F1, hybrid) and pure 129Sv/Ev strain (129S6/SvEv, inbred), respectively, and were purchased from Taconic. Experimental procedures were approved by the Animal Care and Use Committee of Harvard Medical School.

Local Shwartzman Reaction Induction

8- to 12-week-old age-matched male mice were primed on day 0 by a subcutaneous injection of *Escherichia coli* LPS 055:B5 at 100 μg/mouse in 0.1 ml of sterile PBS with a 30G 1/2 gauge needle (Becton Dickinson). 24 hr later (day 1), recombinant murine TNF (R&D) at 0.3 μg/mouse was injected at the same site (Subramaniam et al., 1996). On day 2, the skin was excised and evaluated for MPO activity and/or paraffin sections were made. Hematoxylin and eosin (HE)-stained paraffin sections were prepared, and the degree of inflammatory cell infiltration, thrombus formation, and fibrin deposition as well as hemorrhage were scored microscopically, as described below (Grading of Lesions).

In some experiments, skin air pouches were generated prior to the induction of the LSR. 4 ml of air was injected under the dorsal skin of mice followed by an additional injection of air on day 3. After 7 days, LPS was instilled in the air pouch, followed 24 hr later by injection of TNF. After an additional 24 hr, the air pouch was lavaged with 3 ml ice-cold PBS. Equal amount of lavage fluid containing the same number of cells from mice were assayed for elastase release.

Grading of Lesions

Morphometric Analysis

The skin was excised and paraffin sections were made for microscopic grading of lesions. The sections were hematoxylin and eosin (HE) stained to assess hemorrhaging and the percentage of morphologically intact vessels that were occluded by thrombi. A specific esterase stain described below identified neutrophils, and fibrin deposition was assessed by fibrin/fibrinogen immunostaining. Quantification of hemorrhaging and neutrophil accumulation were done as follows and reported as the percent of total events. For a hemorrhage score, four consecutive fields from the HE-stained sections were obtained at 40× magnification and digitized as JPEG images with QImaging QCapture Software (Quantitative Imaging Corp., Burnaby, BC, Canada). The stored digital images were analyzed by Image-Pro Plus software (Media Cybernetics, Silver Spring, MD), in which the specified color was extracted and total area was measured. The red signal in H&E-stained sections indicated red blood cells and the extracted signal inside vessels was excluded. The blue signal in esterase-stained sections indicated neutrophils. The darker blue, large cells were mast cells and were excluded. The score for each of these was given as a percentage and calculated as follows: red or blue signal divided by the total measured area. Sections were immunostained with antibody to fibrin to assess fibrin localization in the sections, and the percentage of morphologically intact vessels in the entire section with fibrin staining outside the vessel wall was determined.

Neutrophil Enumeration

Chloroacetate Esterase Reaction. Paraffin sections from the Shwartzman lesion site were deparaffinized and incubated in freshly prepared chloroacetate solution containing 0.0125% Naphesol AS-D (Sigma) and 0.0625% Fast Blue BB salt (Sigma) in phosphate buffer (pH 7.3) for 1.5 hr in the dark. Neutrophils were enumerated on digitized images as described in the previous section.

Myeloperoxidase Assay. Skin samples were excised, weighed, and homogenized in 10% hexadecyltrimethylammonium bromide in 50 mM phosphate buffer (pH 6.0). The samples were centrifuged and the supernatant was assayed for MPO activity with a chromogen (o-dianisidine HCl) as previously described (Bradley et al., 1982). The relative MPO activity was converted to neutrophil content in tissues via a standard curve generated with defined numbers of bone marrow-derived murine neutrophils.

Immunohistochemistry

For fibrin staining, paraffin sections from the Shwartzman lesion site were deparaffinized, sequentially blocked with avidin D solution and biotin blocking solution (Vector Laboratories), and stained with a rabbit anti-human fibrinogen (1:1000 dilution; Dako), which cross-reacts with mouse fibrin/fibrinogen. Sections were then treated with a biotinylated goat anti-rabbit antibody (Vector Laboratories) and developed with DAB substrate kit for horseradish peroxidase (Vector Laboratories). Immunostaining for complement C3 was conducted as previously reported with some modification (Sinclair et al., 1981). In brief, 7 μm deparaffinized/hydrated paraffin sections were treated with 0.1% trypsin (Sigma) for 30 min at 37°C to unmask antigen. The sections were incubated with rabbit anti-human C3 complement at 1:100. Staining was visualized by microscopy at 40×.

Ribonuclease Protection Assay

Snap-frozen skin samples from the Shwartzman lesion were homogenized in TRIzol reagent (Life Technologies, Gaithersburg, MD) with a Dounce-type homogenizer. Total RNA was then subjected to a multiprobe RPA system (BD Biosciences, San Diego, CA) with the mCK-2b DNA template set according to the manufacturer's instructions. Protected fragments were resolved in a 6% polyacrylamide-urea gel. The dried gels were developed with a phosphorimager (Molecular Dynamics, Sunnyvale, CA), and bands were quantitated with NIH image. Within each sample, the density of each specific mRNA transcript was divided by that of the glyceraldehyde phosphate dehydrogenase (GAPDH) band.

Neutrophil Adoptive Transfers

Murine neutrophils were collected from two femurs by washing out the bone marrow with 1 ml of ice-cold RPMI with 5% FCS followed by NH_4Cl lysis. Neutrophils were isolated from the cell suspension by density gradient centrifugation on Percoll (Sigma) with stepwise gradients of 55%, 65%, and 75% Percoll. After centrifugation at 1600 rpm for 30 min at 4°C without the brake, the band between 65% and 75% of Percoll was collected. Cells (>95% neutrophils) were then washed and suspended in HBSS without Ca^{2+}/Mg^{2+} supplemented with 0.25 mM HEPES (pH 7.4), at a concentration of 2.5×10^7 cells/ml. 5×10^6 cells in 200 μl HBSS without Ca^{2+}/Mg^{2+} were injected into mice via tail vein 2 hr prior to TNF injection. Assuming a blood volume of 2.2 ml per mouse, the heterologous neutrophils are present at a concentration of approximately 2.3×10^6 neutrophils per ml of blood. The circulating neutrophil count of a wild-type C57Bl/6 mouse averaged 2.5×10^6 neutrophils per ml. Thus, the reconstituted neutrophils are introduced into mice, on average, at a 1:1 ratio with the endogenous circulating neutrophils.

Neutrophil Adhesion Assay

Fresh mouse serum mixed at equal volume with DPBS (PBS plus Ca^{2+} and Mg^{2+}) was placed in 24-well plates and incubated at 37°C for 1 hr. The plates were washed with PBS and blocked with 1% PVP (Polyvinylpyrrolidone, Sigma) at room temperature for 1 hr and washed extensively with PBS at 37°C for 40 min. 2.5×10^5 neutrophils in 500 μl of DPBS, pretreated with rmTNF (50 ng/ml at 37C for 15 min), were added to the wells for 40 min, and then the plates were washed gently three times with prewarmed PBS. The remaining adherent cells in three microscopic fields at 40× magnification were quantified.

Primary Granule Release Assay

Elastase release was analyzed with a real-time fluorimetric assay as previously described (Sklar et al., 1982). In brief, 2.5×10^5 neutrophils in 500 μl DPBS pretreated with TNF were added to 24-well plates coated with fresh mouse serum as described above in the presence of 100 μl 20 μM elastase substrate peptide N-methoxysuccinyl-Ala-Ala-Pro-Val-7-amido-4-methylcoumarin (Sigma). The fluorescence of the samples was measured at 380 nm excitation and 460 nm emission with a Spectra Max spectrofluorimeter (Molecular Devices, Sunnyvale, CA) at 2 min intervals over a period of 60 min total. Elastase activity was calculated as being proportional to the instantaneous slope of the increase in the fluorescence of the reaction product 7 amino 4-methylcoumarin. The percent release of elastase was calculated by dividing the elastase activity by the total cellular elastase activity as measured by lysing cells in the well with 0.02% Triton-X100 at the end of the run. For analysis of neutrophils and lavage fluid retrieved from the air pouch of mice, equal number of cells in lavage fluid were evaluated.

Statistical Analysis

All data are expressed as means ± standard error of the mean (SEM) for data resulting from in vivo analyses of mice and ± standard deviation (SD) for data derived from in vitro assays on isolated murine neutrophils. In all cases, an unpaired t test was used to compare two groups. Significance was set at $p < 0.05$.

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